EXPLORING CRITICAL HOST-PATHOGENS INTERACTIONS DURING CITROBACTER RODENTIUM LETHAL INFECTION IN IMMUNOCOMPROMISED HOSTS

by

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Abstract

Attaching/Effacing(A/E) pathogens include Enteropathogenic *Escherichia coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC), which cause gastroenteritis and are significant causes of foodborne diseases worldwide. *Citrobacter rodentium* (CR), as the murine equivalent, produces the same characteristic A/E lesions. While CR causes self-limiting infection in immunocompetent hosts, the morbidity and mortality are significantly higher in immunocompromised hosts. In the infection cycle of CR, the pathogen senses the changes of external environment and expresses an array of virulence factors. However, the adaptation process and critical host-pathogen interactions under immunocompromised conditions remain elusive. We utilized the infection of wild-type CR in interleukin-22 (*Il-22*) knockout ($Il22^{-/-}$) mice as a model to dissect the crucial host-pathogen interactions during CR infection in immunocompromised hosts associated with severe symptoms and mortality. Previous studies from our lab utilizing RNA-sequencing (RNA-seq) analysis of gene transcription in host-adapted CR derived from the infected $Il22^{-/-}$ mice and *in vitro* culture reveal a substantial number of genes upregulated in the host-adapted CR. Among

them, several genes were significantly upregulated in host-adapted CR, indicating that they might function as important virulence factors accounting for the infection-caused severe symptoms and lethality, including *bioF*, *fepA*, and *Rod_48101*. Here, we generated chromosomal knockout strains of *bioF, fepA*, and *Rod_48101* and assessed the impacts of deletions of these genes on CR proliferation *in vitro* and virulence *in vivo*. Our results demonstrate that deletion of these genes does not impair the proliferation of CR *in vitro*. Meanwhile, infections with Δ*fepA*, Δ*bioF*, and *ΔRod-48101* strains still result in severe body weight loss and high mortality in the infected *Il22*$^{-/-}$ mice, as does by wild-type CR. Therefore, our findings suggest that *bioF, fepA*, and *Rod_48101* is dispensable for CR virulence in the immunocompromised *Il22*$^{-/-}$ mice. The dramatic upregulation of these genes is most likely the consequences of, rather than the causative triggers for the severe inflammatory responses resulting in mortality during CR infection in *Il22*$^{-/-}$ mice. Our results also indicate a complex host-pathogen interactions and bacterial virulence regulatory mechanisms during A/E pathogen infections in immunocompromised hosts associated with severe symptoms and outcomes, which are worthy of further investigations.

Primary reader: Dr. Fengyi Wan

Secondary reader: Dr. Gundula Bosch

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Introduction

Attaching/Effacing (A/E) pathogens; *Citrobacter rodentium* infection

Diarrheagenic pathogens are the second cause of infection related death among children under the age of 5 years (WHO, 2017), and pathogenic *Escherichia coli* is a major contributor[1–3]. While around six pathotypes of pathogenic E. coli have been associated with enteric diseases, Enteropathogenic *Escherichia coli (*EPEC) and Enterohemorrhagic *Escherichia coli* (EHEC) share distinctive virulent mechanisms. Both EHEC and EPEC form characteristic histological lesions on intestinal mucosa, characterized by attachment to the host intestinal epithelial cells (IECs) and effacement of the brush border microvilli [2,4,5]. Formation of A/E lesions requires a unique protein secretion system termed type III secretion system (T3SS), which is mainly encoded in locus of enterocyte effacement (LEE) pathogenicity island[6,7]. The structure of LEE and the function of T3SS effectors have been well described[8]. EHEC/EPEC have around 5000 genes in the genome, but only a small portion of non-T3SS related genes have been functionally related to the A/E pathogenicity.

Naturally, EPEC and EHEC do not infect mice, which brings difficulties in establishing mouse model. This could be the result of competence with other intestinal commensals, since pretreatment with antibiotics enables the colonization of EHEC/EPEC in mice intestinal lumen[9]. The mouse pathogen *Citrobacter rodentium* (CR), which causes transmissible colonic crypt hyperplasia, is the only natural murine A/E pathogen[10]. CR shares 67% of its genes with EHEC/EPEC, including critical virulence factors like LEE island[10], type IV pilus[11], and a type VI secretion system[10]. CR infection forms nearly identical A/E lesions from those caused by EHEC/EPEC in human[12–14], which makes it an ideal model for researching the virulence factors as well as host-pathogen interaction mechanisms of A/E pathogens. Most studies apply an oral inoculation method of laboratory-cultured CR, which robustly results in intestinal infection[15,16]. In 1-3 days post inoculation (DPI), a small portion of CR firstly colonize at the caecal lymphoid patch while the majority just pass through the gastrointestinal tract[17,18]. From 4 DPI, CR starts to adhere to the IECs of distal colon and rectum, where it expresses virulence genes, proliferates, and reach the peak of bacterial load[12,18,19]. From 12 DPI, CR load starts to decrease as colonized IECs are shed into the intestinal lumen[20]. Meanwhile, the adaptive immune response facilitates

bacteria clearance by opsonizing and phagocytosis[21,22]. During the infection cycle, CR triggers an excessive cell proliferation and repair response: pericryptal myofibroblasts secrete R-spondin 2 (RSPO2), which activates the WNT pathway of $LGR5^+$ stem cell[23]. $LGR5^+$ stem cells are the progenitor of IECs, and overactivation of $LGR5^+$ stem cells results in accumulation of undifferentiated transit amplifying cells, which causes crypt elongation and thickening of intestinal mucosa[24,25]. Moreover, these poorly differentiated colonocytes have decreased expression of carbonic anhydrase 4 and HCO_3^- - Cl^- exchanger SLc26a3, which results in disruption of ion exchange and fluid loss[26]. Usually, for immunocompetent C57BL/6 mice, infection of CR leads to mild and self-limited symptoms including diarrhea, hematochezia, and body weight loss[27]. In 2-3 weeks post infection, CR will be completely cleared, and mice develop protection against reinfection eventually[28,29].

Since CR is shed into the intestinal lumen and feces during mid-late stage of infection, it could also be transmitted to other mice in the same litter via coprophagy. Strikingly, after passing through the gastrointestinal tract, CR shows significantly higher infectivity, as it establishes infection with 1000-fold lower CFU[19,30]. Moreover, in the fecal-oral route infection, such "hyperinfectious" pathogens will bypass the caecal lymphoid patches and

directly colonize at the distal colon[29]. These outcomes suggest an adaption process in the caecum, in which bacteria sense the change of external environment and express virulence factors, including T3SS[4]. While the detailed mechanism beneath this adaption is largely unknown, various studies suggest that CR receives multiple signals including variation of temperature[31], host-derived chemicals[32–34], microbiota metabolites[35,36] and mechanical forces [37]. These signals triggers expression of a series of genes, including LEE, which plays a critical role in the initial infection stage. However, it remains elusive whether other genes are involved in the host-adaption process and the mechanism of the regulation of virulence factors is largely unknown.

Role of *Il-22* in host immune response to *C. rodentium* infection

To achieve sterile clearance of CR, both innate and adaptive immune response are required. Briefly, CR infection is first detected by pattern recognition receptors (PRRs) including TLR2, TLR4, TLR9, NOD1, and NOD2[38–41]. These PRRs trigger downstream response including pro-inflammatory cytokine secretion, proliferation of IECs, induction of iNOS,

and recruitment of neutrophils, macrophages and dendritic cells [42–47]. Collectively, these responses restrict the dissemination of the pathogen, and provide critical signals for effector lymphocytes. In the clearance phase, B cell as well as $CD4^+$ T cells are indispensable. T cell activated plasma cells secret IgG, which opsonizes CR for activation of complement system and promotes phagocytosis by neutrophils[21,22,48,49]. Meanwhile, the rest luminal CR is outcompeted by commensals[29], which leads to complete clearance of CR in around 21 days post infection.

Various cytokines are involved in the innate and adaptive immune response against CR infection, including IL-6, IL-12, IFNγ, IL-1β, IL-18, IL-17, IL-23, and IL-22[41,50–54]. Notably, IL-22 is believed to play a critical role in host immune response to CR infection, which is supported by the significantly elevated mortality (nearly 100%) of CR infected *Il-22*$^{-/-}$ mice[27]. During the infection, the major source of IL-22 shifts from type 3 innate lymphoid cells (ILC3s) in the expansion phase[55] to neutrophils in the steady-state phase [56,57]and Th17 and Th22 cells in the clearance phase[58,59]. IL-22 has 2 important functions against CR infection. First, IL-22 directly stimulates IECs cells to secret antimicrobial peptides (AMPs) including S100A family protein, RegIIIβ, and RegIIIγ via interaction with IL-22R[60,61]. Both S100A protein and

RegIIIβ directly inhibit CR by chelating essential nutritional metal ions and biding to lipidA, respectively[62–66]. While no evidence suggests that RegIIIγ protein directly kills CR, studies indicate that RegIIIγ protein has protective effects in CR infection, probably by protecting mice from complicating Gram-positive bacterial infection[27,67]. Meanwhile, IL-22 promotes intestinal integrity by upregulating tight junction protein claudin-1[68] and promoting fucosylation of mucins, which reduces the risk of dissemination and systematic infection[67]. Moreover, some studies indicate that IL-22 is involved in regulation of intestinal microbiota, which could be another mechanism of its anti-CR effect[67,69]. Various studies indicate that patients with autoimmune diseases or chronic HIV infection have lower IL-22 level[70–72], which may renders them more vulnerable to A/E pathogen related infection. However, the virulence regulation mechanism of CR infection in immunocompromised/immunodeficiency host is largely unknown.

Results

Several genes were upregulated in host-adapted *C. rodentium*.

To identify novel regulation factors of CR, we performed RNA-seq on both host-adapted and wild type CR strains. The sequencing data was mapped to CR genome and normalized into RPKM (Reads Per Kilobase Million) values (Fig 1). Compared to wild type CR, host-adapted CR showed significantly higher expression of several genes, including *fepA*, *bioF*, and an unannotated gene *Rod_48101*, hinting that these genes could be potential virulence factors upregulated by CR after adaption to the host intestinal environment.

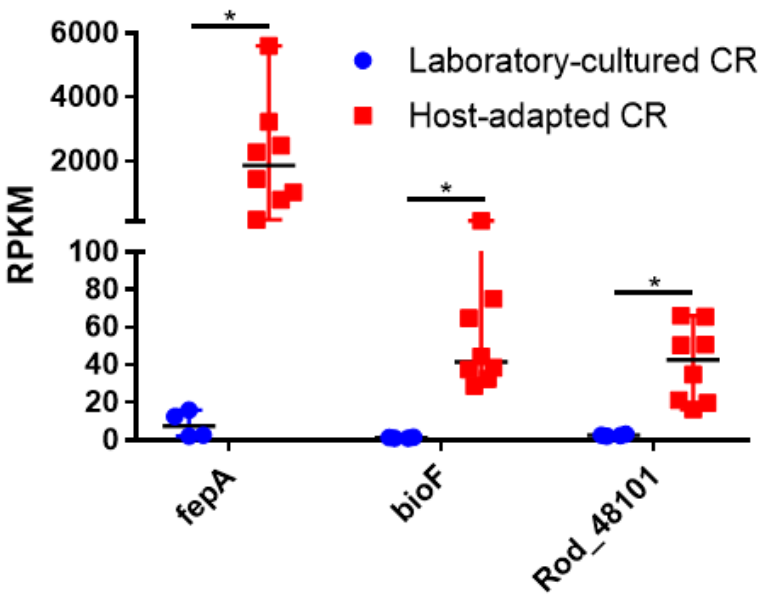

Figure 1 Upregulation of potential virulence factors in host-adapted *C. rodentium*. RNA-seq experiment on host-adapted (n=8) and laboratory-cultured (n=4) CR was performed previously. The raw data of RNA-seq was mapped to the CR genome and normalized into RPKM values. Two-tailed Student's t test was conducted for statistical analysis. (ns, not significant, * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.001$)

Generation of gene-knockout *C. rodentium* strains.

In this study, scarless chromosomal deletion method was utilized to construct mutated CR strains. Primers were designed to amplify the upstream and downstream fragments (homologous arms) of target genes

or to test the presence of junction fragments (Fig 2.1 & Table 2).

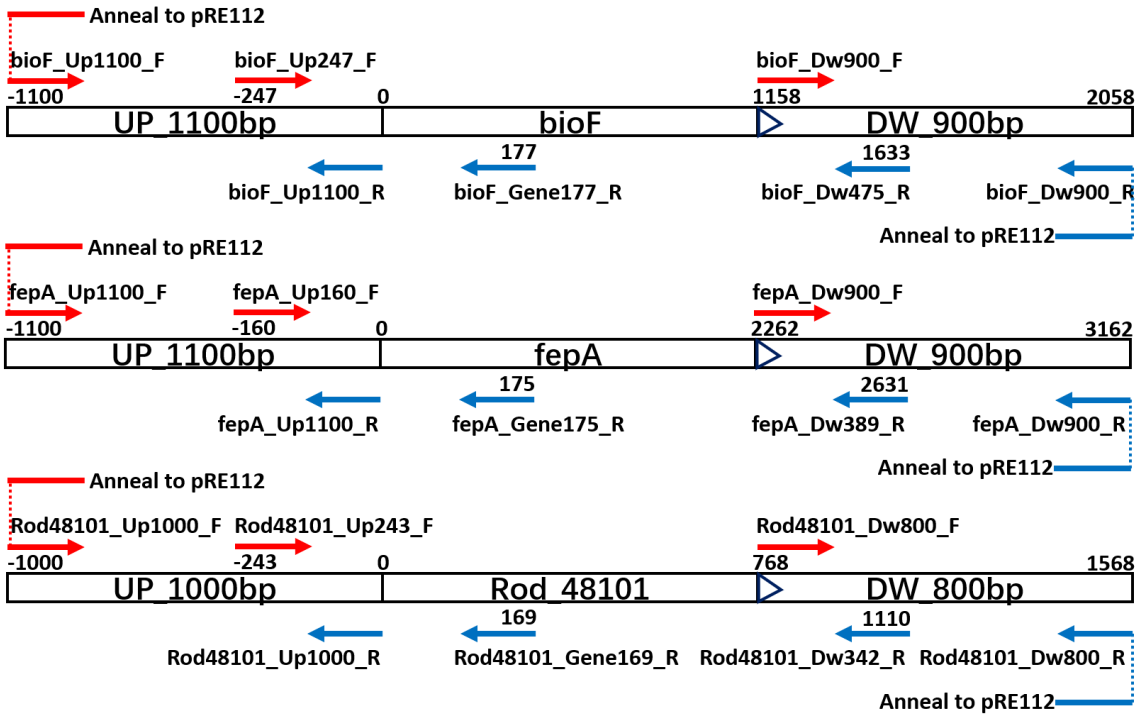

Figure 2 Diagram for primer design in scarless gene knockout 3 sets of primers were used in the scarless gene knockout experiment. The Up 1100/1000-Dw 900/800 primer pairs were used to amplify the homologous arms of target genes and create overlap regions with pRE112. The Up-Dw primer pairs were used to detect the junction region of homologous arms on the recombined vector or mutated genome. The Up-Gene primer pairs were used to detect the presence of target gene on the genome.

PCR-synthesized homologous arms and linearized pRE112 plasmid were conjugated via Gibson Assembly. These vectors were transformed into competent SY327 cells via electroporation, and colonies with Cm resistance

were picked and PCR confirmed to ensure the presence of fusion sequences on pRE112(Fig 3).

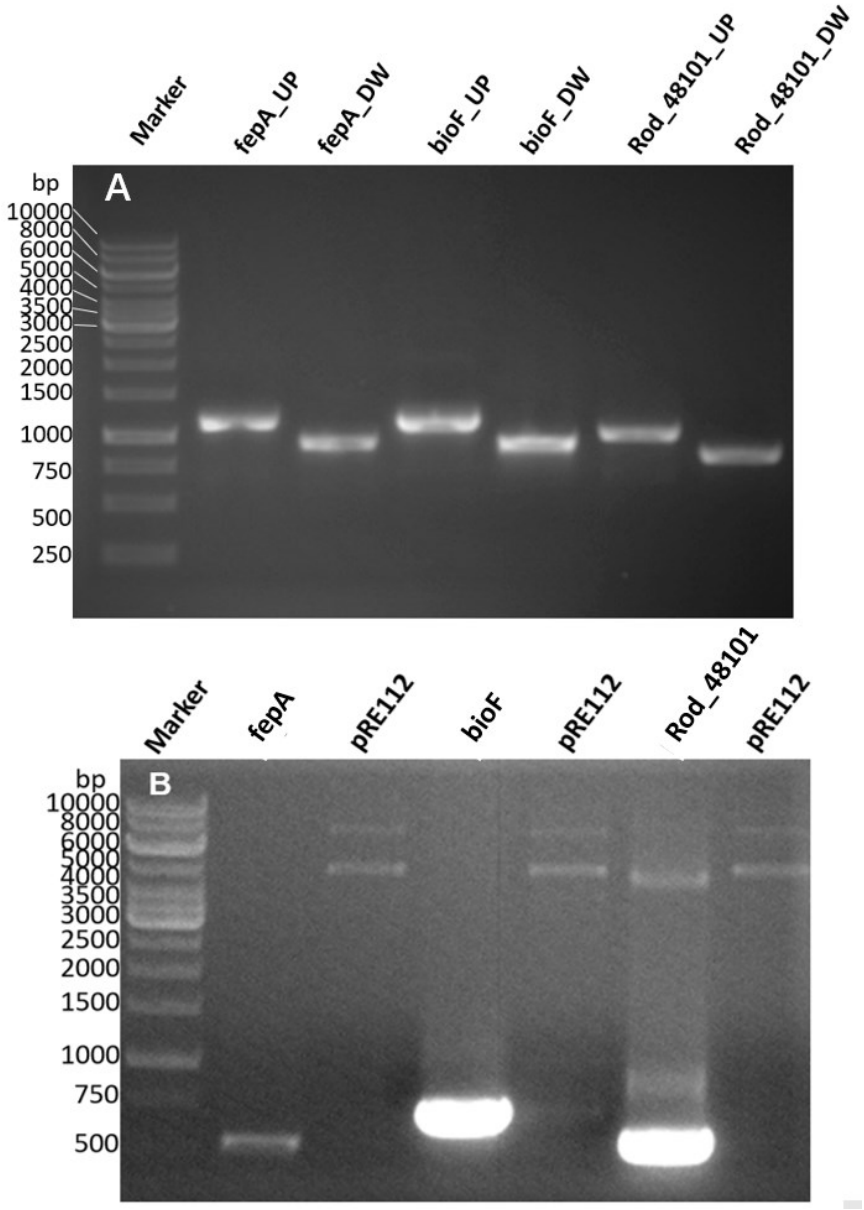

Figure 3 Synthesis of homologous arms and PCR confirmation of Gibson Assembly. (A) The homologous arms of *fepA* (1100/900bp), *bioF* (1100/900 bp), and *Rod_48101* (1000/800bp) were synthesized via PCR and were analyzed in 1% agar gel. (B) After electroporation, colonies with chloramphenicol resistance were cultured in LB broth and the recombined plasmids were extracted, PCR confirmed, and analyzed in 1% agar gel. Original pRE112 was added as a negative control in PCR confirmation.

The vectors were further transferred into wild type CR, and after two rounds of allelic recombination, colonies with only Amp resistance (from helper plasmid pKD46) were cultured and PCR checked to select the target gene knockout strains (Fig 4). All constructs were further verified that no additional mutations were introduced via Sanger sequencing.

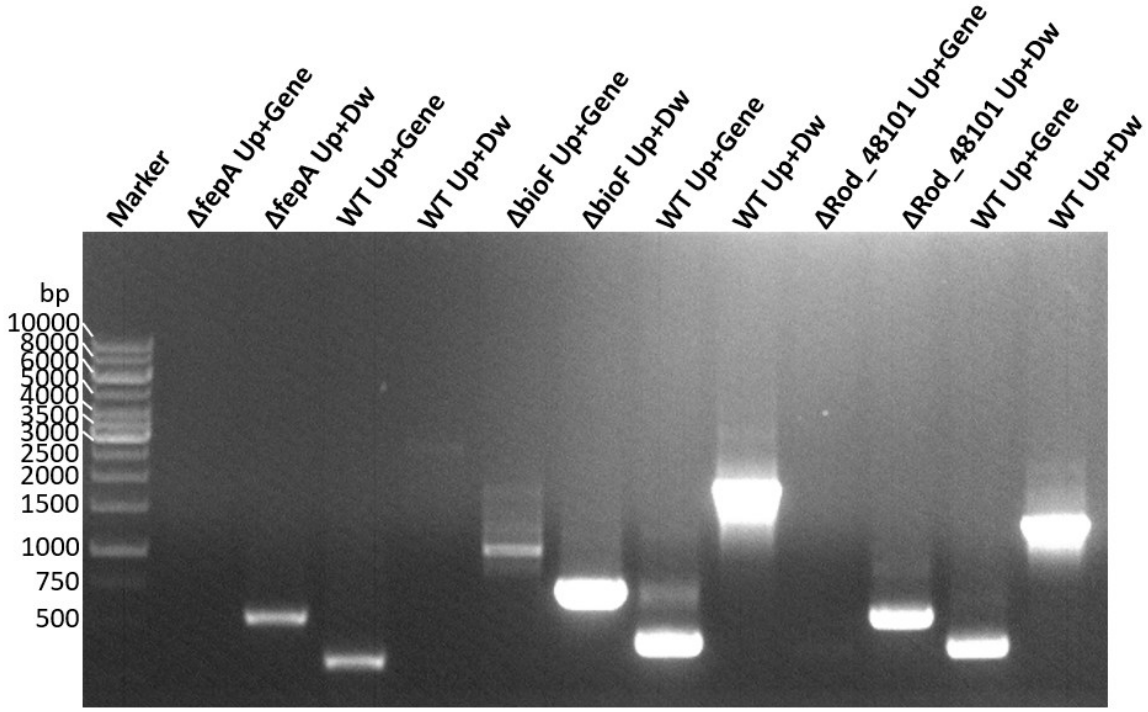

Figure 4 PCR confirmation of gene knockout. Colonies with only ampicillin resistance were cultured and confirmed via PCR, and WT CR was used as a negative control. Two primer pairs were used in the PCR: Up-Dw pair to detect the fusion fragment of up-down sequences, and Up-Gene pair to detect the presence of target gene. Samples with expected PCR product using Up-Dw pair and no product using Up-Gene pair were regarded as positive results.

Deletion of *fepA*, *bioF*, and *Rod_48101* does not impair the proliferation of *C. rodentium in vitro*.

As previous studies showed that *Il-22*$^{-/-}$ mice were extremely vulnerable to wild type CR infection and showed nearly 100% of mortality, we hypothesized that infection by these mutated CR stains would result in decreased severity and mortality, if the correlated genes were involved in pathogenesis. To rule out the possibility that deletion of candidate genes impaired the proliferation of CR, we first measured the growth curve of the three candidates along with WT CR in LB broth. Moreover, Dulbecco's Modified Eagle Medium (DMEM) is proved to strongly induce the expression of T3SS of CR[6,7,29], which could better mimic the "activated" state of CR. Therefore, growth curve was measured in DMEM as well. In both conditions, all three gene knockout strains showed no growth defect compared with WT CR (Fig 5). This result suggests that *bioF*, *fepA* and

Rod_48101 are not directly involved in proliferation, thus the mutant strains are suitable for the following *in vivo* infection experiment.

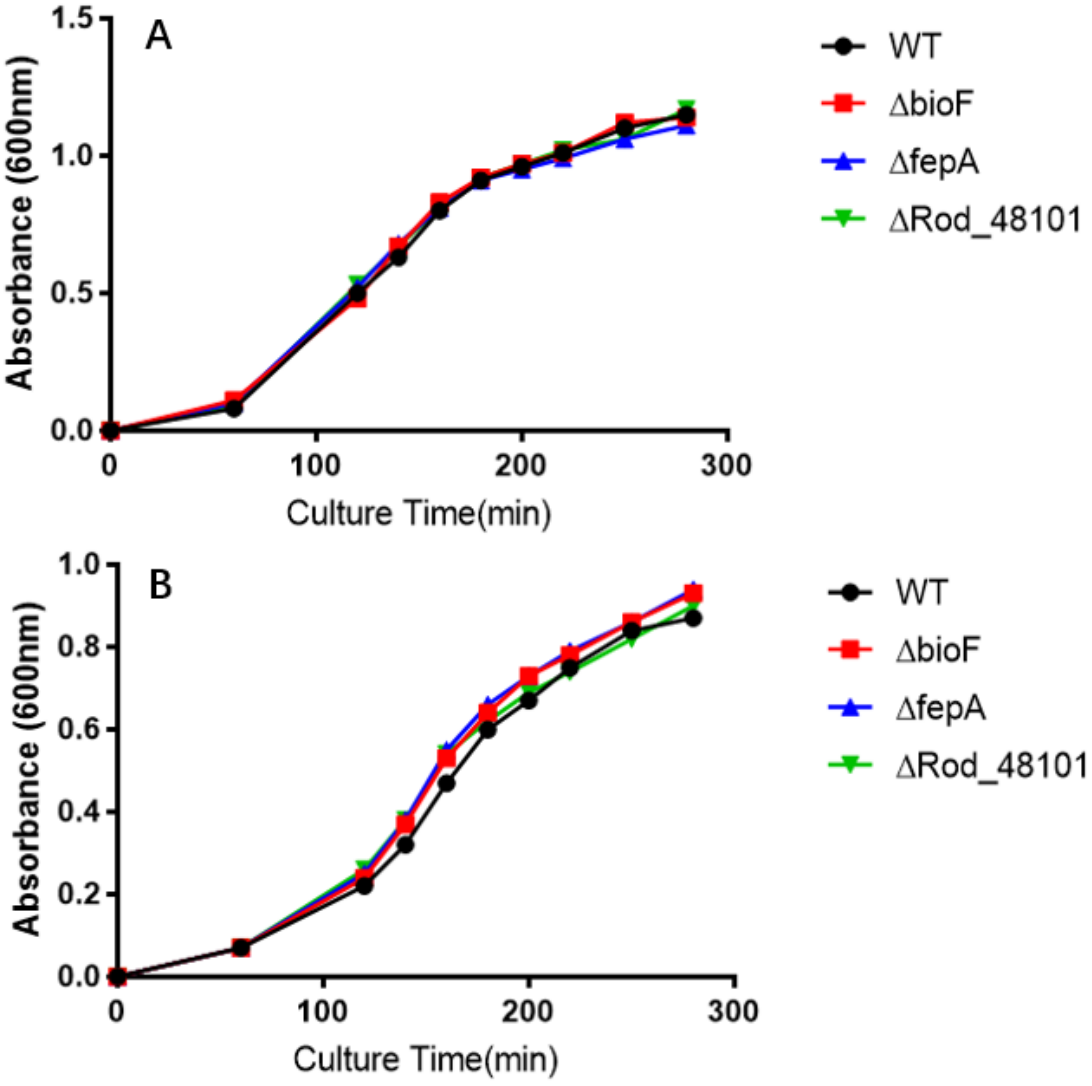

Figure 5 Growth curves of wild type, Δ*bioF*, Δ*fepA*, and Δ*Rod_48101* C. rodentium strains in LB broth (A)and DMEM(B). Growth curves of indicated CR strains in LB medium (A) and Dulbecco's Modified Eagle's Medium(B), at 1:50 dilution from normalized overnight culture.

fepA, _bioF_, and _Rod_48101_ are dispensable for C. rodentium induced lethal infection on _Il-22_$^{-/-}$ mice.

We carried *in vivo* infection experiments to assess the pathogenetic capacity of Δ*bioF*, Δ*fepA*, Δ*Rod_48101*, and WT CR strains on *Il-22*$^{-/-}$ mice. Clinical manifestations, body weight change, and fecal CR CFU were monitored after inoculation. All four groups of mice manifested typical CR infection symptoms: diarrhea, dehydration, hematochezia, and significant weight loss (Fig 6). There were no significant differences in relative body weight loss between WT CR and three CR mutants at all time spots (Fig 7).

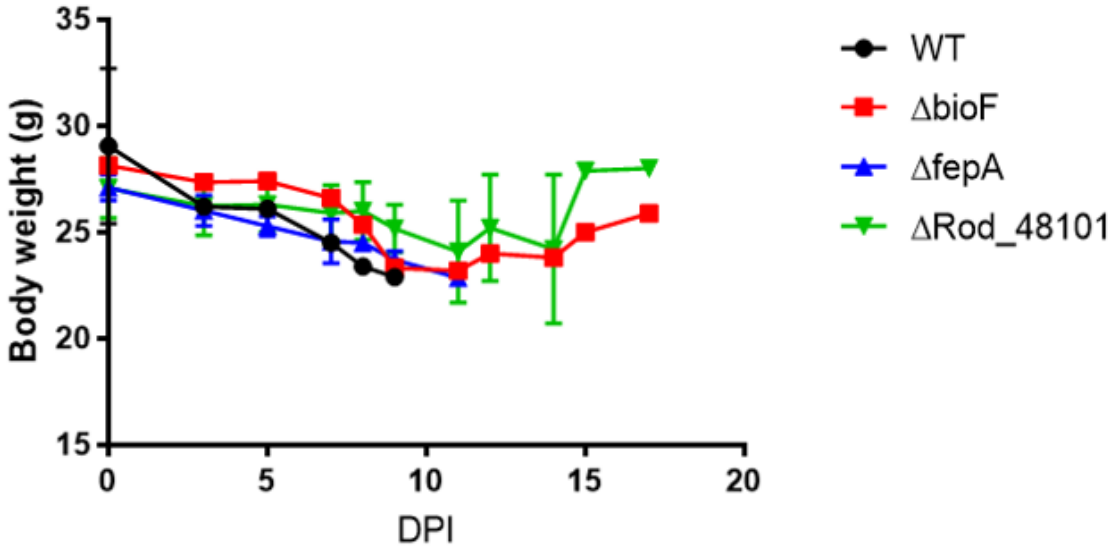

Figure 6 Body weight loss of wild type, Δ*bioF*, Δ*fepA*, and Δ*Rod_48101 C. rodentium* infected mice. Weight loss of *Il-22*$^{-/-}$ mice at indicated time post inoculation with wild type, Δ*bioF*, Δ*fepA*, or Δ*Rod_48101* CR.

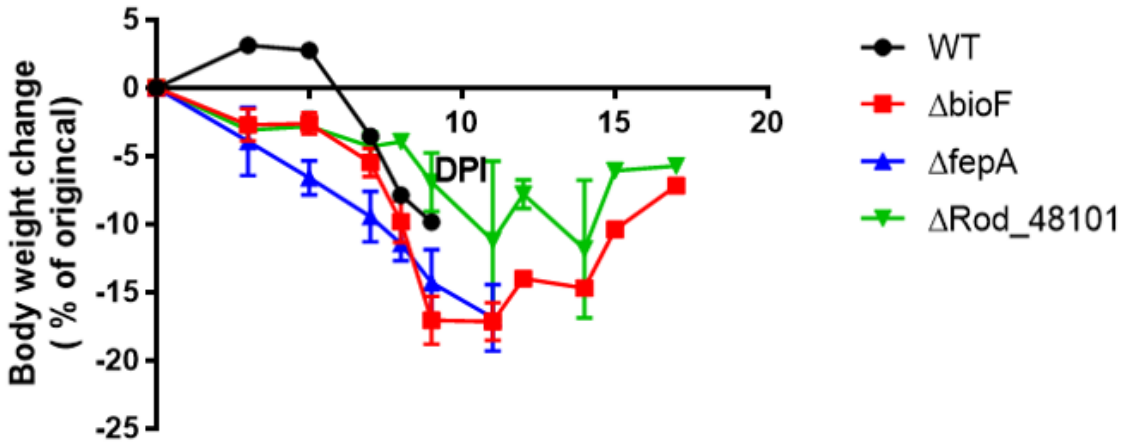

Figure 7 Relative body weight loss of Wild Type, Δ*bioF*, Δ*fepA*, and Δ*Rod_48101 C. rodentium* infected mice. Relative body weight loss of *Il-22*$^{-/-}$ mice at indicated time post inoculation with wild type, Δ*bioF*, Δ*fepA*, or Δ*Rod_48101* CR.

Consistently, WT CR infection showed 100% lethality by 14 DPI and the median survival time was 7 days. The median survival time of Δ*bioF* and Δ*fepA* group showed no significant difference with WT group, whereas Δ*Rod_48101* groups showed attenuated mortality and significantly longer

median survival time (Fig 8).

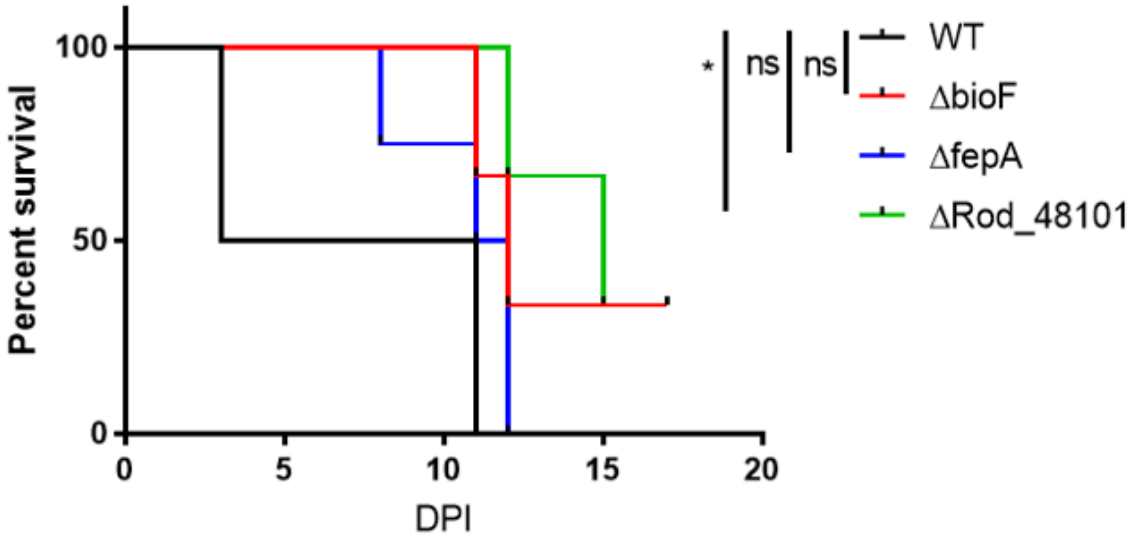

Figure 8 Survival curve of wild type, Δ*bioF*, Δ*fepA*, and Δ*Rod_48101 C. rodentium* infected mice. Log-rank analysis of the survival rate in *Il-22*$^{-/-}$ mice inoculated with wild type, Δ*bioF*, Δ*fepA*, or Δ*Rod_48101* CR. ns, not significant; * p<0.05.

Correspondingly, Δ*Rod_48101* CR infected mice showed 100-1000 fold lower fecal CR CFUs compared with WT or Δ*bioF*/Δ*fepA* CR infected groups (Fig 9). Such outcome implied that *Rod_48101* might serve as a potential virulence factor.

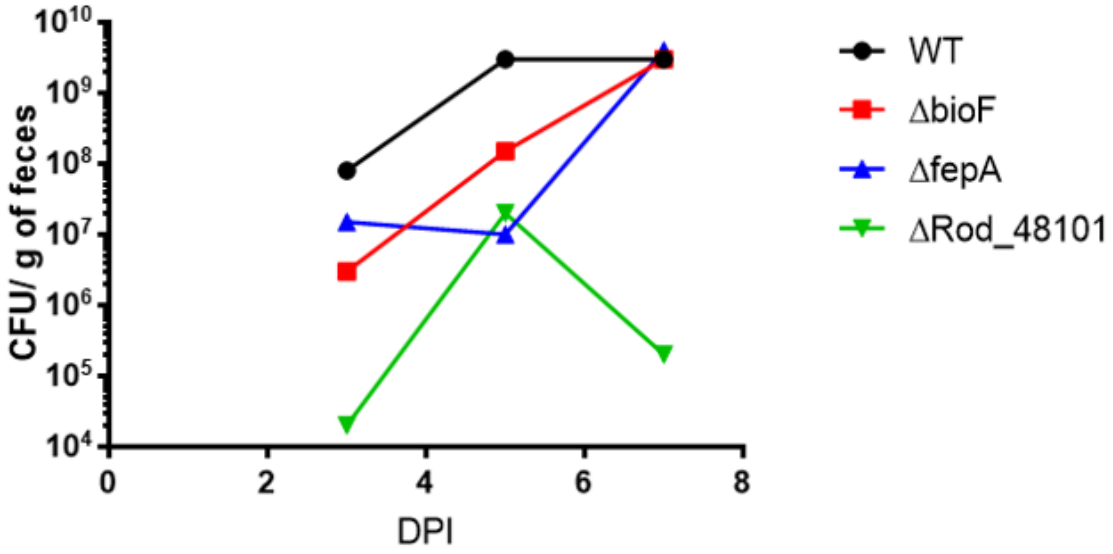

Figure 9 Fecal *C. rodentium* Load of wild type, Δ*bioF*, Δ*fepA*, and Δ*Rod_48101 C. rodentium* infected mice. Fecal samples were obtained at indicated time post inoculation with wild type, Δ*bioF*, Δ*fepA*, and Δ*Rod_48101* CR. Samples were serial diluted in sterile PBS and plated on MacConkey plates. The CFU values of each sample were normalized to the mass of the feces.

To further ascertain this result, we carried a repetitive *in vivo* infection experiment of Δ*Rod_48101* CR strain. WT CR served as a negative control and A12F6 mutated CR strain served as a positive control, which showed no mortality in previous experiments. Infection with Δ*Rod_48101* CR still caused severe weight loss as WT CR did. The median survival time and mortality at 14 DPI of WT and Δ*Rod_48101* CR group showed no difference. On the contrary, infection with A12F6 CR only led minor body weight fluctuation and no mortality was observed even in 15 DPI (Fig 10&11&12). Thus, our results suggested that *fepA*, *bioF*, and *Rod_48101* were

dispensable for CR induced mortality on *Il-22*$^{-/-}$ mice. However, these genes might still be involved in CR host-pathogen interaction and regulation of virulence (see Discussion).

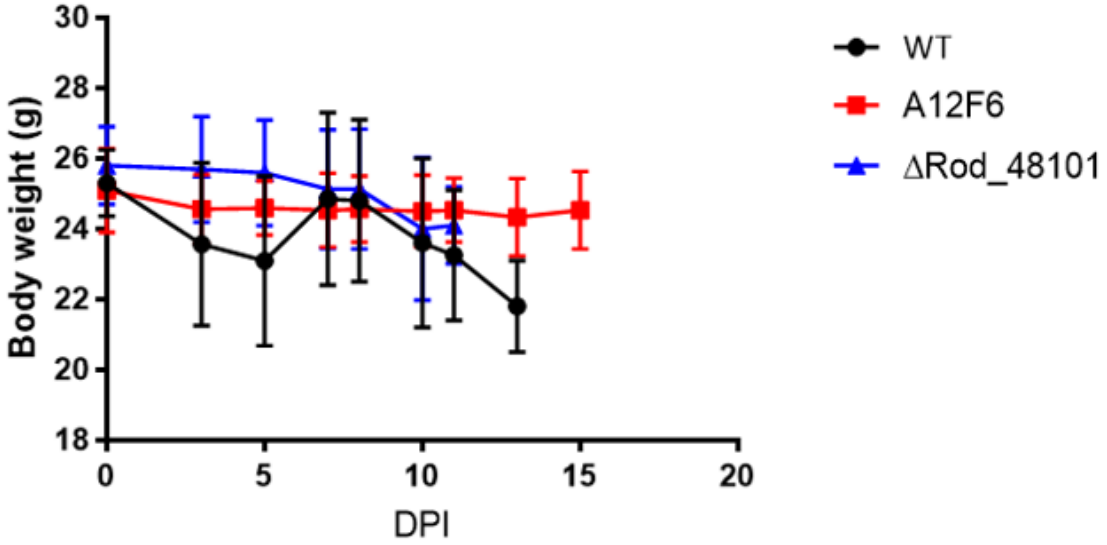

Figure 10 Body weight loss of wild type, A12F6, and Δ*Rod_48101 C. rodentium* infected mice (Repetitive experiment). Weight loss of *Il-22*$^{-/-}$ mice at indicated time post inoculation with wild type, A12F6, or Δ*Rod_48101* CR.

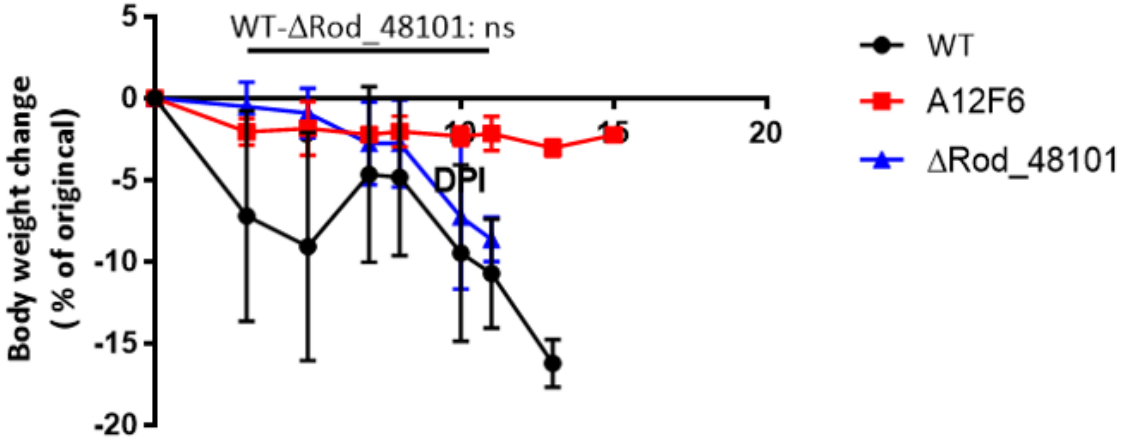

Figure 11 Relative Body weight loss of wild type, A12F6, and Δ*Rod_48101 C. rodentium* infected mice (Repetitive experiment). Relative weight loss of *Il-22*$^{-/-}$ mice at indicated time post inoculation with wild type, A12F6, or Δ*Rod_48101* CR. ns, not significant.

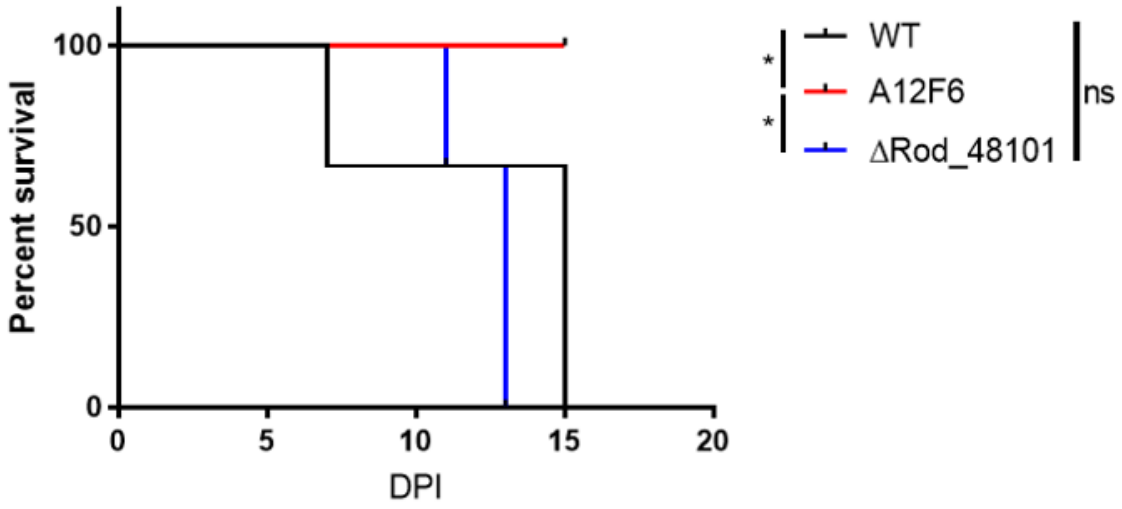

Figure 12 Survival curve of wild type, A12F6, and Δ*Rod_48101 C. rodentium* infected mice (Repetitive experiment). Log-rank analysis of the survival rate in *Il-22*$^{-/-}$ mice inoculated with wild type, A12F6, or Δ*Rod_48101* CR. ns, not significant; * p<0.05.

Discussion

While *Citrobacter rodentium* infection creates a good animal model to investigate the pathogenesis of A/E pathogens EHEC and EPEC, the host-pathogen interactions in immunocompromised/immunodeficient hosts remains largely unknown. Moreover, a recent study indicates that the circulating IL-22 levels in infants are lower thus they are more susceptible to severe infection[73], which just further increases the urgency. Here we demonstrate that *bioF*, *fepA*, and *Rod_48101*, while are significantly upregulated in the pathogenesis of CR, are not essential virulence factors of CR. Deletion of these genes could neither impair the proliferation and colonization of CR nor alleviate the body weight loss or reduce the mortality in infected Il-22$^{-/-}$ hosts. Although we did observe a significant change in the survival curve of Δ*Rod_48101* CR infected mice in the first round of infection, we believe that this significance could simply be attributed to the unusual survival of the last mouse in this group. Several studies suggest that, except for the virulence capacity of CR, genetic background and composition of intestinal microbiota also influence the outcome of CR infection, which may account for the individual

differences[23,74,75]. The survival curves and body weight change curves of this research also suggest that some individuals are more resistant/vulnerable to CR infection. Therefore, a large sample size should be applied in the future to diminish the bias introduced by individual differences.

It remains unknown why these genes were significantly upregulated in host-adapted CR. Since the absence of these genes did not impair the virulence capacity of CR, we hypothesize that they are involved in redundant pathways that could be compensated by systems with similar function. *bioF* encodes a highly conserved 8-amino-7-oxononanoate synthase, which catalyzes the first step of biotin biosynthesis[76]. Biotin acts as an essential cofactor for carboxylases and decarboxylase. While human could only acquire biotin from external source, microorganisms are able to either synthesize biotin *de novo* or absorb it from the environment[77]. This bi-phase system is strictly regulated by biotin protein ligase BirA, which acts as a biotin metabolism mediator as well as a negative regulator of biotin synthetic operon. When the supplement of biotin exceeds the demand, biotin-BirA complex binds to the biotin operator and suppress the

transcription of the operon, which contains *bioF*[78–80]. This sensing and regulation mechanism plays an important role in CR pathogenesis. Humans absorb biotin majorly in the small intestine. This absorption is highly effective, which results in a lower environmental biotin concentration in the small intestine than in the colon[81]. Since we obtained host-adapted CR in the distal colon, it is reasonable that host-adapted CR has a higher expression level of *bioF* than those obtained from a biotin-rich environment. Moreover, Bin Yang reported that BirA is negatively associated with T3SS via fur (ferric uptake regulation protein) in EHEC. This mechanism ensures that A/E pathogens express T3SS and attach to the epithelial cells in the large intestine, which is more hostile[35]. The outer membrane protein *fepA*, which serves as the receptor of siderophore ferric enterobactin, is a key component of bacterial iron uptake system[82]. Since the cellular concentration of free ferric ions is usually too low for bacteria to survive, bacteria evolved different mechanisms to obtain iron from different iron-binding protein. Enterobactin is a high affinity iron chelator secreted by CR to overcome the limitation of iron supply. Enterobactin could from a high stable coordinate compound with iron, which enables bacteria to sequester the iron from host protein[83]. The complex, termed

FeEnt, is recognized by membrane protein FepA and transported into the bacteria[84,85]. This mechanism may explain the upregulation of *fepA* in host-adapted CR. Meanwhile, in the host immune response to CR, IL-22 induces secretion of multiple bactericidal proteins, including lipocalin-2, which could sequester iron from siderophores (bacterial iron chelating agents, like enterobactin)[63,86]. This mechanism, together with the fact that CR has additional iron transporting mechanisms other than *fepA*-enterobactin, may explain our findings that *fepA* knockout CR strains could still proliferate and cause death in IL-22 deficiency mice[82,87]. Finally, although *Rod_48101* has not been annotated so far, homology analysis suggests that it is a putative siderophore-interacting protein[88]. Therefore, the upregulation of *Rod_48101* could be attributed to the activation of iron uptake system.

Taken together, our data shows that *bioF*, *fepA*, and *Rod_48101*, while are significantly upregulated in host-adapted CR, are not indispensable virulence factors in the pathogenesis of CR on Il-22$^{-/-}$ mice. Our findings indicate a complex host-pathogen interactions and bacterial virulence regulatory mechanisms during A/E pathogen infections in

immunocompromised hosts, and more study is required to fully understand it.

Material and Methods

Ethics statement

All animal experiments were performed according to protocol number MO19H269, approved by the Johns Hopkins University's Animal Care and Use Committee. *Il-22* knockout (*Il-22*$^{-/-}$) mice in C57Bl/6 background were maintained in a specific pathogen-free facility and fed autoclaved food and water *ad libitum*.

Cell culture, reagents, and plasmids

Wild type, Δ*bioF*, Δ*fepA*, and Δ*Rod_48101 C. rodentium* strains were grown in LB broth at 37℃ overnight with shaking. Chloramphenicol (Cm, 25mg/L) and ampicillin (Amp, 100mg/L) LB agar plates were used to select positive strains. Salt free LB agar plates with 5% sucrose were used to selected colonies that underwent secondary recombinant. Suicidal plasmid pRE112 was used as the vector, which contains a chloramphenicol resistance gene CmR as a selective marker, a SacB gene for secondary recombination and SacI/KpnI cleavage sites. Restrictive endonucleases SacI and KpnI were used to digest pRE112 in CutSmart™ buffer (New England BioLabs, Ipswich, MA).

Constructing gene-knockout CR strains

Δ*bioF*, Δ*fepA*, and Δ*Rod_48101* CR strains were generated using a scarless gene deletion method via SacB-based allelic exchange. A suicidal vector, pRE112, was linearized by digestion of restriction endonuclease SacI and KpnI. Homologous arms of target genes were generated by high-fidelity PCR from WT CR genomic DNA using LiSpark^{TM} SuFi PCR Master Mix (LifeSct, Rockville, MD) and were assembled with the linearized vector using Gibson Assembly Master Mix (New England BioLabs, Ipswich, MA). The recombined vector was transformed into SY327 using a Bio-Rad MicroPulser (Bio-Rad Laboratories, Hercules, CA) and positive colonies were selected using Cm containing LB agar plates. The colonies were PCR confirmed using 2xLiTaq^{TM} PCR Master Mix (LifeSct, Rockville, MD). The recombined pRE112 was extracted and transformed into SM10 via heat shock, which later conjugated with CR and passed the vector. The colonies were cultured and plated on Cm LB agar plates, where they underwent the first allelic exchange to incorporate the vector. Cm-resistant CR colonies were cultured and plated on salt free LB agar plates with sucrose, where they underwent

the secondary allelic exchange to expel the SacB gene. Colonies with only Amp resistance were PCR confirmed for the Up-Down conjugation part, and desired strains were Sanger sequenced to get rid of additional mutations.

Growth curve measurement

Wild type, Δ*bioF*, Δ*fepA*, and Δ*Rod_48101 C. rodentium* strains were grown in LB broth at 37℃ overnight with shaking. The medium was normalized to OD_{600}=1 and was diluted at 1:50 and grown at 37℃ with shaking. 1ml of *C. rodentium* culture was taken at indicated time to read OD_{600} value in a 96-well plate with a CO8000 Cell Density Meter (Biochrom, UK).

CR infection in mice

Male *Il-22* knockout mice (12-18 weeks) were fasted for 6 hours before inoculation. Wild type, Δ*bioF*, Δ*fepA*, and Δ*Rod_48101 C. rodentium* strains were grown in LB broth at 37℃ overnight with shaking. The medium was normalized to OD_{600}=1, and then concentrated by 10 times in PBS. Each mouse was orally inoculated with 200 µl of PBS containing indicated *C.*

rodentium strains. Mice were observed once in two days for weight loss and mortality.

Bacterial counts

Stool samples were collected at indicated times post inoculation. Samples were homogenized and diluted in PBS (100µl PBS for 10mg stool). The supernatant was serial diluted and plated on MacConkey plates, incubated overnight at 37℃. C. rodentium CFUs were calculated the following day.

Statistical analysis

All statistical analysis was performed with GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA). Mean with standard errors of means (S.E.M.) or median with range were plotted in graphs and were detailed in figure legends. Statistical analysis on survival curves was performed using the log-rank (Mantel-Cox) test. Statistical analysis on gene expression levels and relative body weight change was performed using Student's t test. Significant differences were considered: ns, non-significant difference; * at $p < 0.05$; ** at $p < 0.01$; *** at $p < 0.001$; **** at $p < 0.0001$.

Supplementary Tables

Table 1 Sequence and function of primers used in this study.

Name	Sequence	Function
BioF_up1100_F	caagcttcttctagaggtacACCAAATTAAA AACATTTAGGTTTACGAGTCTAC	Amplify BioF homolog ous arms
BioF_up1100_R	b	
BioF_dw900_F	cggcagcagtACAGGCCATTGCGGCGGC	
BioF_dw900_R	catgaattcccgggagagctGGGCGTCGCT ATTGCGCTG	
FepA_UP1100_F	caagcttcttctagaggtacAAGCTTTGTCCC GCCACG	Amplify FepA homolog ous arms
FepA_UP1100_R	tgcactggcaTGTTTATCCTGCTTTTCTTT AGCCAC	
FepA_DW900_F	aggataaacaTGCCAGTGCACTCTCCCG	
FepA_DW900_R	catgaattcccgggagagctCCCTGAAGGGT TGCAAAGTG	
ROD_48101_up1000_F	caagcttcttctagaggtacCGGCATGGTGA GCAACGTC	Amplify ROD_48 101 homolog ous arms
ROD_48101_up1000_R	cagttaaacgTTTTTGCCTTCGTGAAAAA ATCAGATATATCG	
ROD_48101_dw800_F	aaggcaaaaaCGTTTAACTGCCCGATAA C	
ROD_48101_dw800_R	catgaattcccgggagagctATATTTTTCAAT CATCACCTGC	
BioF_Up247F	gtcgcgagcagatgaatg	PCR confirma tion for Gibson Assembl y and gene knockou t
BioF_Gene177R	gataatatgcggatggcggc	
BioF_Dw475R	gcgattcgcatgatgctgg	
FepA_Up160F	caatagcgtattgtcgcgctg	
FepA_Gene175R	gaatttcatcggcggtgatg	
FepA_Dw389R	cggcgtaaaccaagcttc	
ROD_48101_Up243F	ccttcatgatgatgtcgcac	
ROD_48101_Gene169R	ctttgcagtgatcgtcgaag	
ROD_48101_Dw342R	gaaaagctatactagcgacagc	

Table 2 Mice body weight change in the first round of infection

Animal #	Sex	GT	strain	0 (orignal)	3	5	7
L	M	IL22 KO	WT	32.70	dead		
R	M	IL22 KO	WT	25.40	26.20	26.10	24.50
L	M	IL22 KO	ΔBioF	27.90	26.80	26.90	25.90
R	M	IL22 KO	ΔBioF	28.40	27.30	27.50	26.80
NC	M	IL22 KO	ΔBioF	28.10	28.00	27.80	27.10
L	M	IL22 KO	ΔFepA	27.00	27.60	26.10	24.80
R	M	IL22 KO	ΔFepA	28.00	26.80	25.70	25.90
NC	M	IL22 KO	ΔFepA	28.00	25.20	25.50	26.00
LR	M	IL22 KO	ΔFepA	25.40	24.50	23.90	21.60
L	M	IL22 KO	ΔROD_48101	26.60	25.90	25.90	26.00
R	M	IL22 KO	ΔROD_48101	24.90	24.00	24.30	23.60
NC	M	IL22 KO	ΔROD_48101	29.70	28.80	28.70	28.10

strain	8	9	11	12	14	15	17
WT							
WT	23.40	22.90	dead				
ΔBioF	25.30	24.10	23.50	24.00	23.80	25.00	25.90
ΔBioF	24.80	22.90	dead				
ΔBioF	26.00	23.00	22.90	dead			
ΔFepA	24.30	24.40	23.10	dead			
ΔFepA	24.10	23.00	dead				
ΔFepA	25.10	23.70	22.60	dead			
ΔFepA	dead						
ΔROD_48101	25.40	23.90	20.60	dead			
ΔROD_48101	24.00	24.20	23.00	22.70	20.70	dead	
ΔROD_48101	28.60	27.40	28.70	27.70	27.70	27.90	28.00

Table 3 Mice body weight change in the second round of infection

Animal #	Sex	GT	strain	0	3	5
L	M	IL22KO	WT	23.90	19.10	18.40
R	M	IL22KO	WT	24.90	24.80	24.50
NC	M	IL22KO	WT	27.10	26.80	26.40
L	M	IL22KO	ΔFepA&EntD	27.00	26.20	25.70
R	M	IL22KO	ΔFepA&EntD	22.90	22.80	23.10
NC	M	IL22KO	ΔFepA&EntD	25.40	24.70	25.00
L	M	IL22KO	ΔROD_48101	28.00	28.70	28.60
R	M	IL22KO	ΔROD_48101	24.80	24.30	24.20
NC	M	IL22KO	ΔROD_48101	24.60	24.10	24.00

strain	7	8	10	11	13	15
WT	dead					
WT	22.40	22.50	21.20	21.40	20.50	dead
WT	27.30	27.10	26.00	25.10	23.10	dead
ΔFepA&EntD	26.20	26.00	26.10	25.90	26.20	26.30
ΔFepA&EntD	22.60	22.80	22.60	22.80	22.40	22.50
ΔFepA&EntD	24.80	24.90	24.80	24.90	24.40	24.80
ΔROD_48101	28.40	28.40	27.70	25.20	dead	
ΔROD_48101	24.20	24.30	23.60	23.00	dead	
ΔROD_48101	22.80	22.70	20.70	dead		

Table 4 PCR protocols

PCR Recipe for Homologous Arms Amplification	
Reagents	**Volume(μl)**
2x LiSpark Max Buffer	12.5
dNTP Mix	0.5
Template DNA	1
Forward primer	1
Reverse primer	1
LiSpark Max SuFi DNA polymerase	0.5
Sterile water	8.5
Total	25
PCR protocol	
Temperature(℃)	**Time**
(1) 95	5:00
(2) 95	0:15
(3) 55-70 (depends on primers)	0:15
(4) 72	2:00
Back to (2) for 34 times	
(5) 72	0:30
(6) 4	∞

PCR Recipe for Confirmation	
Reagents	**Volume(µl)**
2x MyTaq Matser Mix	10
Template DNA	2 for plasmid; 5 for bacterial culture
Forward primer	1
Reverse primer	1
Sterile water	6 for plasmid;3 for bacterial culture
Total	20
PCR protocol	
Temperature(℃)	**Time**
(1) 95	8:00
(2) 95	0:15
(3) 56	0:15
(4) 72	2:00
Back to (2) for 27 times	
(5) 72	0:30
(6) 4	∞

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Curriculum Vitae

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EDUCATION

Johns Hopkins University, Bloomberg School of Public Health Baltimore, MD, US (2019-Present)

-Degree: ScM (Master of Science)

-Major: Molecular Microbiology & Immunology

Fudan University, School of Life Sciences, Shanghai, China (2015-2019)

-Degree: Bachelor of Science

-Major: Biological Sciences

RESEARCH EXPERIENCES

The virulence regulation of bacterial pathogen *Citrobacter rodentium* in immunocompromised mice (01/2020-Present)

Advisor: Prof. Fengyi Wan, Dept. of Biochemistry & Molecular Biology, JHSPH

- Constructed plasmids for scarless gene knockout in *C. rodentium*.
- Generated 7 gene knockout *C. rodentium* strains.
- Assess the impacts of candidate gene deletions on CR infection caused mortality and clinical manifestation in interleukin 22 knockout (*Il22*$^{-/-}$) mice.
- Constructing a library of Tn5-inserted *C. rodentium* mutants and screening for novel zinc transporter genes.

Oncogenic FAP Positive Fibroblasts Promote Intrahepatic Cholangiocarcinoma (ICC) via MDSC Recruitment (09/2018-07/2019)

Advisor: Prof. Rui He, Dept. of Immunology, School of Basic Medical Sciences, Fudan U

- Illustrated that FAP positive cancer associated fibroblasts (CAFs) play a critical role in promoting tumor growth of ICC via myeloid-derived suppressor cells (MDSCs) using siRNA-mediated gene knock-down and cell co-culturing system.
- Identified *ALOX5* as the key gene upregulated in CAFs-activated MDSCs and MDSCs-derived LTB4 as a key mediator in maintaining tumor stemness in ICC microenvironment via NGS analysis, WB, ELISA and cell co-culture.
- Identified BLT2 as the major surface receptor of LTB4 on ICC cells and mTOR activation following LTB4-BLT2 interaction via NGS analysis, specific antagonist blocking and WB.
- Hypothesized IL6 and IL33 as potential pro-tumor cytokines secreted by FAP positive CAFs stimulated MDSCs via NGS analysis and GSEA. (Validated by subsequent work)
- Participated in identifying CCL2 as a key promoter secreted by FAP positive CAFs to enhance the pro-tumor effect of MDSCs.

Separation and Proliferation of Mouse Tumor Infiltrating Lymphocytes (TILs) and Research on Its Antineoplastic Activity (09/2017-06/2018)

Advisor: Prof. Naishuo Zhu, Dept. of Microbiology, School of Life Science, Fudan U

- Fundamental training for molecular biology, immunology, and animal experiments.
- Separated TILs from tumor-bearing mice via MACS. Preliminary research on its anti-neoplastic capacity after PD-L1 antibody treatment.

Summer Research Program: Training for Bioinformatics in Stem Cell Research (06/2018-08/2018)

Advisor: Dr. Bing Zhang, Harvard University. (Present: Prof. in Westlake University)

- Transcriptome analysis (RNA-seq data) of skin stem cells with Cufflinks, Salmon and other bioinformatics tools on R and Linux.

Summer Research Program: Research and Modification on Magnetotactic Bacteria (08/2018-09/2018)

Advisor: Prof. Wei Lin, Institute of Geology and Geophysics, Chinese Academy of Science

- Cultured magnetotactic bacteria from natural environment with self-created device. Modified magnetosome of MB with cobalt and research on its chemical and magnetic property.

PUBLICATION

[1] Yuli Lin, Bingji Li, Xuguang Yang, Qian Cai, Weiren Liu, Mengxin Tian, **Haoyang Luo**, Wei Yin, Yan Song, Yinghong Shi, Rui He. (2019) Fibroblastic FAP promotes intrahepatic cholangiocarcinoma growth via MDSCs recruitment ***Neoplasia*** 21(12):1133-1142.

[2] Yuli Lin; Qian Cai; Yu Chen; Tiancong Shi; Weiren Liu; Li Mao; Bo Deng; Zhen Ying; Yuan Gao; **Haoyang Luo**; Xuguang Yang; Xiaowu Huang; Yinghong Shi; Rui He. (2020) Fibrotic microenvironment shapes CD33+ MDSCs to promote cancer stemness via hyperactivated 5-LO/LTB4-BLT2 signaling in intrahepatic cholangiocarcinoma. ***Immunity*** (Submitted)